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## A CELLULASE PREPARATION

## FIELD OF INVENTION

The present invention concerns a cellulase preparation comprising a single-component endoglucanase, a detergent additive comprising the cellulase preparation, a detergent composition containing the cellulase preparation as well as methods of treating cellulose-containing fabrics with the cellulase preparation.

## BACKGROUND OF THE INVENTION

10 It is well known in the art that repeated washing of cotton-containing fabrics generally causes a pronounced, unpleasant harshness in the fabric, and several methods for overcoming this problem have previously been suggested in the art. For example GB 1,368,599 of Unilever Ltd. teaches the use  
15 of cellulytic enzymes for reducing the harshness of cotton-containing fabrics. Also, US 4,435,307 (of Novo Industri A/S) teaches the use of a cellulytic enzyme derived from Humicola insolens as well as a fraction thereof, designated AC<sub>x</sub>I, as a harshness reducing detergent additive. Other uses of cellulytic  
20 enzymes mentioned in the art involve soil removal from and colour clarification of fabric (cf. for instance EP 220 016), providing increasing water absorption (JP-B-52-48236) and providing a localized variation in colour to give the treated fabrics a "stone-washed" appearance (EP 307,564). Cellulytic  
25 enzymes may furthermore be used in the brewing industry for the degradation of  $\beta$ -glucans, in the baking industry for improving the properties of flour, in paper pulp processing for removing the non-crystalline parts of cellulose, thus increasing the proportion of crystalline cellulose in the pulp, and for  
30 improving the drainage properties of pulp, and in animal feed for improving the digestibility of glucans.

The practical exploitation of cellulytic enzymes has, to some extent, been set back by the nature of the known

cellulase preparations which are often complex mixtures. It is difficult to optimise the production of multiple enzyme systems and thus to implement industrial cost-effective production of cellulytic enzymes, and their actual use has been hampered by 5 difficulties arising from the need to apply rather large quantities of the cellulytic enzymes to achieve the desired effect on cellulosic fabrics.

The drawbacks of previously suggested cellulase preparations may be remedied by using preparations comprising 10 a higher amount of endoglucanases. A cellulase preparation enriched in endoglucanase activity is disclosed in WO 89/00069.

#### SUMMARY OF THE INVENTION

A single endoglucanase component has now been isolated which exhibits favourable activity levels relative to 15 cellulose-containing materials.

Accordingly, the present invention relates to a cellulase preparation consisting essentially of a homogenous endoglucanase component which is immunoreactive with an antibody raised against a highly purified ~43 kD endoglucanase 20 derived from Humicola insolens, DSM 1800, or which is homologous to said ~43 kD endoglucanase.

The finding that this particular endoglucanase component of cellulase is advantageous for the treatment of cellulose-containing materials is of considerable practical significance: it permits a cost-effective production of the cellulase, e.g. by employing recombinant DNA techniques for producing the active component, and makes the actual effective application of the enzyme feasible in that a smaller quantity of the cellulase preparation is requested to produce the desired 30 effect on cellulosic materials.

#### DETAILED DISCLOSURE OF THE INVENTION

The cellulase preparation of the invention is advantageously one in which the endoglucanase component exhibits a

CMC-endoase activity of at least about 50 CMC-endoase units per mg of total protein.

In the present context, the term "CMC-endoase activity" refers to the endoglucanase activity of the endoglucanase component in terms of its ability to degrade cellulose to glucose, cellobiose and triose, as determined by a viscosity decrease of a solution of carboxymethyl cellulose (CMC) after incubation with the cellulase preparation of the invention, as described in detail below.

10 Preferred cellulase preparations of the invention are those in which the endoglucanase component exhibits a CMC-endoase activity of at least about 60, in particular at least about 90, CMC-endoase units per mg of total protein. In particular, a preferred endoglucanase component exhibits a CMC-15 endoase activity of at least 100 CMC-endoase units per mg of total protein.

The CMC-endoase (endoglucanase) activity can be determined from the viscosity decrease of CMC, as follows:

A substrate solution is prepared, containing 35 g/l CMC 20 (Hercules 7 LFD) in 0.1 M tris buffer at pH 9.0. The enzyme sample to be analyzed is dissolved in the same buffer.

10 ml substrate solution and 0.5 ml enzyme solution are mixed and transferred to a viscosimeter (e.g. Haake VT 181, NV sensor, 181 rpm), thermostated at 40°C.

25 Viscosity readings are taken as soon as possible after mixing and again 30 minutes later. The amount of enzyme that reduces the viscosity to one half under these conditions is defined as 1 unit of CMC-endoase activity.

SDS polyacrylamide gel electrophoresis (SDS-PAGE) and 30 isoelectric focusing with marker proteins in a manner known to persons skilled in the art were used to determine the molecular weight and isoelectric point (pI), respectively, of the endoglucanase component in the cellulase preparation of the invention. In this way, the molecular weight of a specific 35 endoglucanase component was determined to be  $\approx$  43 kD. The isoelectric point of this endoglucanase was determined to be about 5.1. The immunochemical characterization of the

endoglucanase was carried out substantially as described in WO 89/00069, establishing that the endoglucanase is immunoreactive with an antibody raised against highly purified ~43 kD endoglucanase from Humicola insolens, DSM 1800. The cellobio-  
5 hydrolase activity may be defined as the activity towards cellobiose p-nitrophenyl. The activity is determined as  $\mu$ mole nitrophenyl released per minute at 37°C and pH 7.0. The present endoglucanase component was found to have essentially no cellobiohydrolase activity.

10       The endoglucanase component in the cellulase preparation of the invention has initially been isolated by extensive purification procedures, i.a. involving reverse phase HPLC purification of a crude H. insolens cellulase mixture according to US 4,435,307 (cf. Example 1 below). This procedure  
15 has surprisingly resulted in the isolation of a ~43 kD endoglucanase as a single component with unexpectedly favourable properties due to a surprisingly high endoglucanase activity.

          In another aspect, the present invention relates to an  
20 enzyme exhibiting endoglucanase activity (in the following referred to as an "endoglucanase enzyme"); which enzyme has the amino acid sequence shown in the appended Sequence Listing ID#2, or a homologue thereof exhibiting endoglucanase activity. In the present context, the term "homologue" is intended to  
25 indicate a polypeptide encoded by DNA which hybridizes to the same probe as the DNA coding for the endoglucanase enzyme with this amino acid sequence under certain specified conditions (such as presoaking in 5xSSC and prehybridizing for 1 h at ~40°C in a solution of 20% formamide, 5xDenhardt's solution, 50  
30 mM sodium phosphate, pH 6.8, and 50  $\mu$ g of denatured sonicated calf thymus DNA, followed by hybridization in the same solution supplemented with 100  $\mu$ M ATP for 18 h at ~40°C). The term is intended to include derivatives of the aforementioned sequence obtained by addition of one or more amino acid residues to  
35 either or both the C- and N-terminal of the native sequence, substitution of one or more amino acid residues at one or more sites in the native sequence, deletion of one or more amino

acid residues at either or both ends of the native amino acid sequence or at one or more sites within the native sequence, or insertion of one or more amino acid residues at one or more sites in the native sequence.

5           The endoglucanase enzyme of the invention may be one producible by species of Humicola such as Humicola insolens e.g strain DSM 1800, deposited on 1 October 1981 at the Deutsche Sammlung von Mikroorganismen, Mascheroder Weg 1B, D-3300 Braunschweig, FRG, in accordance with the provisions of the Budapest Treaty on the International Recognition of the Deposit of  
10   Microorganisms for the Purposes of Patent Procedure (the Budapest Treaty).

          In a further aspect, the present invention relates to an endoglucanase enzyme which has the amino acid sequence shown  
15   in the appended Sequence Listing ID#4, or a homologue thereof (as defined above) exhibiting endoglucanase activity. Said endoglucanase enzyme may be one producible by a species of Fusarium, such as Fusarium oxysporum, e.g. strain DSM 2672, deposited on 6 June 1983 at the Deutsche Sammlung von  
20   Mikroorganismen, Mascheroder Weg 1B, D-3300 Braunschweig, FRG, in accordance with the provisions of the Budapest Treaty.

          Furthermore, it is contemplated that homologous endoglucanases may be derived from other microorganisms producing cellulolytic enzymes, e.g. species of Trichoderma,  
25   Myceliophthora, Phanerochaete, Schizophyllum, Penicillium, Aspergillus, and Geotricum.

          The present invention also relates to a DNA construct comprising a DNA sequence encoding an endoglucanase enzyme as described above, or a precursor form of the enzyme. In  
30   particular, the DNA construct has a DNA sequence as shown in the appended Sequence Listings ID#1 or ID#3, or a modification thereof. Examples of suitable modifications of the DNA sequence are nucleotide substitutions which do not give rise to another amino acid sequence of the endoglucanase, but which correspond  
35   to the codon usage of the host organism into which the DNA construct is introduced or nucleotide substitutions which do give rise to a different amino acid sequence and therefore,

possibly, a different protein structure which might give rise to an endoglucanase mutant with different properties than the native enzyme. Other examples of possible modifications are insertion of one or more nucleotides into the sequence, 5 addition of one or more nucleotides at either end of the sequence, or deletion of one or more nucleotides at either end or within the sequence.

The DNA construct of the invention encoding the endoglucanase enzyme may be prepared synthetically by 10 established standard methods, e.g. the phosphoamidite method described by S.L. Beaucage and M.H. Caruthers, Tetrahedron Letters 22, 1981, pp. 1859-1869, or the method described by Matthes et al., EMBO Journal 3, 1984, pp. 801-805. According to the phosphoamidite method, oligonucleotides are synthesized, 15 e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in suitable vectors.

A DNA construct encoding the endoglucanase enzyme or a precursor thereof may, for instance, be isolated by establishing a cDNA or genomic library of a cellulase-producing 20 microorganism, such as Humicola insolens, DSM 1800, and screening for positive clones by conventional procedures such as by hybridization using oligonucleotide probes synthesized on the basis of the full or partial amino acid sequence of the endoglucanase in accordance with standard techniques (cf. 25 Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd. Ed., Cold Spring Harbor, 1989), or by selecting for clones expressing the appropriate enzyme activity (i.e. CMC-endoase activity as defined above), or by selecting for clones producing a protein which is reactive with an antibody against 30 a native cellulase (endoglucanase).

Finally, the DNA construct may be of mixed synthetic and genomic, mixed synthetic and cDNA or mixed genomic and cDNA origin prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate), the fragments corresponding to 35 various parts of the entire DNA construct, in accordance with standard techniques. The DNA construct may also be prepared by polymerase chain reaction using specific primers, for instance

as described in US 4,683,202 or R.K. Saiki et al., Science 239, 1988, pp. 487-491.

The invention further relates to a recombinant expression vector into which the DNA construct of the invention 5 is inserted. This may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an 10 extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has 15 been integrated.

In the vector, the DNA sequence encoding the endoglucanase should be operably connected to a suitable promoter and terminator sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell 20 of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. The procedures used to ligate the DNA sequences coding for the endoglucanase, the promoter and the terminator, respectively, and to insert them into suitable vectors are well known to 25 persons skilled in the art (cf., for instance, Sambrook et al., op.cit.).

The invention also relates to a host cell which is transformed with the DNA construct or the expression vector of the invention. The host cell may for instance belong to a 30 species of Aspergillus, most preferably Aspergillus oryzae or Aspergillus niger. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known per se. The use of Aspergillus as a host 35 microorganism is described in EP 238,023 (of Novo Industri A/S), the contents of which are hereby incorporated by ref-



erence. The host cell may also be a yeast cell, e.g. a strain of Saccharomyces cerevisiae.

Alternatively, the host organism may be a bacterium, in particular strains of Streptomyces and Bacillus, and E. coli.  
5 The transformation of bacterial cells may be performed according to conventional methods, e.g. as described in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, 1989.

The present invention further relates to a process for  
10 producing an endoglucanase enzyme of the invention, the process comprising culturing a a host cell as described above in a suitable culture medium under conditions permitting the expression of the endoglucanase enzyme, and recovering the endoglucanase enzyme from the culture. The medium used to  
15 culture the transformed host cells may be any conventional medium suitable for growing the host cells in question. The expressed endoglucanase may conveniently be secreted into the culture medium and may be recovered therefrom by well-known procedures including separating the cells from the medium by  
20 centrifugation or filtration, precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

By employing recombinant DNA techniques as indicated  
25 above, techniques of protein purification, techniques of fermentation and mutation or other techniques which are well known in the art, it is possible to provide endoglucanases of a high purity.

The cellulase preparation or endoglucanase enzyme of the  
30 invention may conveniently be added to cellulose-containing fabrics together with other detergent materials during soaking, washing or rinsing operations. Accordingly, in another aspect, the invention relates to a detergent additive comprising the cellulase preparation or endoglucanase enzyme of  
35 the invention. The detergent additive may suitably be in the form of a non-dusting granulate, stabilized liquid or protected enzyme. Non-dusting granulates may be produced e.g. according

to US 4,106,991 and 4,661,452 (both to Novo Industri A/S) and may optionally be coated by methods known in the art. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, 5 lactic acid or boric acid according to established methods. Other enzyme stabilizers are well known in the art. Protected enzymes may be prepared according to the method disclosed in EP 238,216.

The detergent additive may suitably contain 1 - 500, 10 preferably 5 - 250, most preferably 10 - 100 mg of enzyme protein per gram of the additive. It will be understood that the detergent additive may further include one or more other enzymes, such as a protease, lipase, peroxidase or amylase, conventionally included in detergent additives.

15 According to the invention, it has been found that when the protease is one which has a higher degree of specificity than Bacillus lentus serine protease, an increased storage stability of the endoglucanase enzyme is obtained. (For the present purpose, a protease with a higher degree of specificity 20 than B. lentus serine protease is one which degrades human insulin to fewer components than does the B. lentus serine protease under the following conditions: 0.5 ml of a 1 mg/ml solution of human insulin in B and R buffer, pH 9.5, is incubated with 75  $\mu$ l enzyme solution of 0.6 CPU [cf. Novo 25 Nordisk Analysis Methods No. AF 228/1] per litre for 120 min. at 37°C, and the reaction is quenched with 50  $\mu$ l 1N HCl). Examples of such proteases are subtilisin Novo or a variant thereof (e.g. a variant described in US 4,914,031), a protease derivable from Nocardia dassonvillei NRRL 18133 (described in 30 WO 88/03947), a serine protease specific for glutamic and aspartic acid, producible by Bacillus licheniformis (this protease is described in detail in co-pending International patent application No. PCT/DK91/00067), or a trypsin-like protease producible by Fusarium sp. DSM 2672 (this protease is 35 described in detail in WO 89/06270).

In a still further aspect, the invention relates to a detergent composition comprising the cellulase preparation or endoglucanase enzyme of the invention.

Detergent compositions of the invention additionally  
5 comprise surfactants which may be of the anionic, non-ionic, cationic, amphoteric, or zwitterionic type as well as mixtures of these surfactant classes. Typical examples of anionic surfactants are linear alkyl benzene sulfonates (LAS), alpha olefin sulfonates (AOS), alcohol ethoxy sulfates (AES) and  
10 alkali metal salts of natural fatty acids. It has, however, been observed that the endoglucanase is less stable in the presence of anionic detergents and that, on the other hand, it is more stable in the presence of non-ionic detergents or certain polymeric compounds such as polyvinylpyrrolidone,  
15 polyethylene glycol or polyvinyl alcohol. Consequently, the detergent composition may contain a low concentration of anionic detergent and/or a certain amount of non-ionic detergent or stabilising polymer as indicated above.

Detergent compositions of the invention may contain  
20 other detergent ingredients known in the art as e.g. builders, bleaching agents, bleach activators, anti-corrosion agents, sequestering agents, anti soil-redeposition agents, perfumes, enzyme stabilizers, etc.

The detergent composition of the invention may be  
25 formulated in any convenient form, e.g. as a powder or liquid. The enzyme may be stabilized in a liquid detergent by inclusion of enzyme stabilizers as indicated above. Usually, the pH of a solution of the detergent composition of the invention will be 7-12 and in some instances 7.0-10.5. Other detergent enzymes  
30 such as proteases, lipases or amylases may be included the detergent compositions of the invention, either separately or in a combined additive as described above.

The softening, soil removal and colour clarification effects obtainable by means of the cellulase preparation of the  
35 invention generally require a concentration of the cellulase preparation in the washing solution of 0.0001 - 100, preferably 0.0005 - 60, and most preferably 0.01 - 20 mg of enzyme

protein per liter. The detergent composition of the invention is typically employed in concentrations of 0.5 - 20 g/l in the washing solution. In general, it is most convenient to add the detergent additive in amounts of 0.1 - 5% w/w or, preferably, 5 in amounts of 0.2 - 2% of the detergent composition.

In a still further aspect, the present invention relates to a method of reducing the rate at which cellulose-containing fabrics become harsh or of reducing the harshness of cellulose-containing fabrics, the method comprising treating 10 cellulose-containing fabrics with a cellulase preparation or endoglucanase enzyme as described above. The present invention further relates to a method providing colour clarification of coloured cellulose-containing fabrics, the method comprising treating coloured cellulose-containing fabrics with a cellulase 15 preparation or endoglucanase, and a method of providing a localized variation in colour of coloured cellulose-containing fabrics, the method comprising treating coloured cellulose-containing fabrics with a cellulase preparation or endoglucanase of the invention. The methods of the invention 20 may be carried out by treating cellulose-containing fabrics during washing. However, if desired, treatment of the fabrics may also be carried out during soaking or rinsing or simply by adding the cellulase preparation or the endoglucanase enzyme to water in which the fabrics are or will be immersed.

25 According to the invention, it has been found that the drainage properties of paper pulp may be significantly improved by treatment with the endoglucanase of the invention without any significant concurrent loss of strength. Consequently, the present invention further relates to a method of improving the 30 drainage properties of pulp, the method comprising treating paper pulp with a cellulase preparation or an endoglucanase enzyme according to the invention. Examples of pulps which may be treated by this method are waste paper pulp, recycled cardboard pulp, kraft pulp, sulphite pulp, or thermomechanical 35 pulp and other high-yield pulps.

The present invention is described in further detail with reference to currently preferred embodiments in the fol-

lowing examples which are not intended to limit the scope of the invention in any way.

## EXAMPLES

Example 1Isolation of a ~43 kD endoglucanase from Humicola insolens

1. Preparation of a rabbit antibody reactive with a ~43 kD  
5 endoglucanase purified from Humicola insolens cellulase mixture

Cellulase was produced by cultivating Humicola insolens  
DSM 1800, as described in US 4,435,307, Example 6. The crude  
cellulase was recovered from the culture broth by filtration on  
diatomaceous earth, ultrafiltration and freeze-drying of the  
10 retentate, cf. Examples 1 and 6 of US 4,435,307.

The crude cellulase was purified as described in WO  
89/09259, resulting in the fraction F1P1C2 which was used for  
the immunization of mice. The immunization was carried out 5  
times at bi-weekly intervals, each time using 25 µg protein  
15 including Freund's Adjuvant.

Hybridoma cell lines were established as described in  
Ed Harlow and David Lane, Antibodies. A Laboratory Manual, Cold  
Spring Harbor Laboratory 1988. The procedure may briefly be  
described as follows:

20 After bleeding the mouse and showing that the mouse  
serum reacts with proteins present in the F1P1C2 fraction, the  
spleen was removed and homogenized and then mixed with PEG and  
Fox-river myeloma cells from Hyclone, Utah, USA.

The hybridomas were selected according to the estab-  
25 lished HAT screening procedure.

The recloned hybridoma cell lines were stabilized. The  
antibodies produced by these cell lines were screened and  
selected for belonging to the IgG1 subclass using a commercial  
mouse monoclonal typing kit from Serotec, Oxford, England.  
30 Positive antibodies were then screened for reactivity with  
F1P1C2 in a conventional ELISA, resulting in the selection of  
F4, F15 and F41 as they were all very good in ELISA response  
but were found to have different response in immunoblotting

using Celluzyme (crude H. insolens, DSM 1800, cellulase) in SDS-PAGE followed by Western Blot, indicating that they recognized different epitopes.

The three antibodies were produced in large quantities in the ascites fluid of CRBF<sub>1</sub> mice. The mouse gammaglobulin was purified from ascites fluid by protein A purification using protein A coupled to Sepharose (Kem.En.Tek., Copenhagen, Denmark).

The different monoclonal gammaglobulins were tested for response in a sandwich ELISA using each monoclonal antibody as the catching antibody, various HPLC fractions of Celluzyme as the antigen, and a rabbit antibody raised against endoglucanase B from Celluzyme as the detection antibody.

To visualize binding in the ELISA, a porcine antibody against rabbit IgG covalently coupled to peroxidase from Dakopatts (Copenhagen, Denmark) and was visualized with OPD(1,2-phenylenediamine, dihydrochloride)/H<sub>2</sub>O<sub>2</sub>.

The highest ELISA response was obtained with the monoclonal antibody F41 which was therefore used in the immunoaffinity purification steps.

The purified mouse gammaglobulin F41 was coupled to 43 g of CNBr-activated Sepharose 4B as described by the manufacturer (Pharmacia, Sweden) followed by washing.

## 2. Immunoaffinity purification of ~43 kD endoglucanase from a H. insolens cellulase mixture

H. insolens cellulase mixture (as described above) was diluted to 3% dry matter, and the pH was adjusted to 3.5 in 15 min. at 4 °C. The precipitate was removed by filtration after adjusting the pH to 7.5. Then sodium sulphate was added to precipitate the active enzyme and this was done at 40°C (260 gram per kg at pH 5.5). The precipitate was solubilized with water and filtrated. The acid treatment was repeated. Finally, the product was filtrated and concentrated by ultrafiltration using a polyvinylsulphonate membrane with a 10.000 Mw cut-off.

The cellulase product was then diluted to 3% dry matter, adjusting the pH to 9.0, and subjected to anion exchange

chromatography on a DEAE-Sepharose column as recommended by the manufacturer (Pharmacia, Sweden).

The protease-free cellulase product was applied on the F 41 gammaglobulin-coupled Sepharose column described above at 5 pH 8.0 in sodium phosphate buffer.

After application the column was washed with the same buffer containing 0.5 M sodium chloride. The column was then washed with 0.1 M sodium acetate buffer containing 0.5 M sodium chloride, pH 4.5, after which the column was washed in 5 mM 10 sodium acetate buffer, pH 4.5. Finally, the 43 kD endoglucanase was eluted with 0.1 M citric acid.

Total yield: 25 mg with an endoglucanase activity of 1563 CMC-endoase units.

The eluted protein migrates as a single band in SDS-15 PAGE with an apparent MW of 43 kD and a pI after isoelectric focusing of about 5.0 to 5.2.

Inactive protein was removed by reverse phase purification.

Inactive and active protein was separated by HPLC using 20 a gradient of 2-propanol. Inactive protein elutes at about 25% 2-propanol and the active 43 kD endoglucanase elutes at 30% 2-propanol, the active endoglucanase being detectable by a CMC-Congo Red clearing zone.

In this way, a total of 0.78 mg active protein was 25 recovered with 122 CMC endoase units. This procedure was repeated 30 times.

The 43 kD endoglucanase was recovered by first freeze-drying to remove the TFA and propanol and then solubilizing in phosphate buffer.

30 The endoglucanase activity of the purified material was 156 CMC-endoase units per mg protein and the total yield including freeze-drying was 65% of the endoglucanase activity.

The thus obtained ~43 kD enzyme was used to immunise rabbits according to the procedure described by N. Axelsen et 35 al. in A Manual of Quantitative Immunoelectrophoresis, Blackwell Scientific Publications, 1973, Chapter 23. Purified immunoglobulins were recovered from the antisera by ammonium



sulphate precipitation followed by dialysis and ion exchange chromatography on DEAE-Sephadex in a manner known per se. Binding of purified immunoglobulin to the endoglucanase was determined, and the rabbit immunoglobulin AS 169 was selected for further studies.

**2. Characterization of the ~43 kD endoglucanase:**

Amino acid composition: Using total hydrolysis, the following composition was obtained after amino acid analysis:

|        |    |
|--------|----|
| Asp    | 17 |
| 10 Asn | 15 |
| Thr    | 25 |
| Ser    | 29 |
| Glu    | 6  |
| Gln    | 13 |
| 15 Pro | 21 |
| Gly    | 32 |
| Ala    | 23 |
| Cys    | 20 |
| Val    | 14 |
| 20 Met | 1  |
| Ile    | 7  |
| Leu    | 8  |
| Tyr    | 6  |
| Phe    | 15 |
| 25 Lys | 9  |
| His    | 2  |
| Trp    | 9  |
| Arg    | 12 |

The Mw of the non-glycosylated protein was estimated to be 30,069 based on the amino acid composition. The glycosylation was measured to

|           |    |
|-----------|----|
| Galactose | 10 |
| Mannose   | 28 |

corresponding to a Mw of 6,840, resulting in a total Mw of the endoglucanase of 36,900 (+/- 2,400). The extinction coefficient per mole was estimated as follows:

|   |            |          |                 |
|---|------------|----------|-----------------|
|   | Tryptophan | 9 times  | 5690            |
| 5 | Tyrosine   | 6 times  | 1280            |
|   | Cysteins   | 20 times | 120             |
|   | total      |          | 61290 per mole. |

Extinction coefficients are 1.66 at 280 nm corresponding to 1 mg protein per ml. (Reference: S.C.Gill and P. Hippel, Anal. Biochemistry 182, 312-326 (1989).)

The amino acid sequence was determined on an Applied Biosystems 475A Protein Sequenator using Edman degradation. Only one sequence indicated the purity of the protein. The amino acid sequence is shown in the appended Sequence Listing ID#2.

#### Enzyme properties:

The enzyme is stable between pH 3 and 9.5.

The enzyme does not degrade highly crystalline cellulose or the substrate cellobiose  $\beta$ -p-nitrophenyl, (Cello- biohydrolase substrate), but degrades amorphous cellulose mainly to cellobiose, cellotriose and cellotetraose, indicating that the enzyme may be used to produce cellodextrins from insoluble amorphous cellulose.

The enzyme is active between pH 6.0 and 10.0 with a maximum activity at about 50°C.

#### Example 2

#### Cloning and expression of the ~43 kD endoglucanase in *Aspergillus oryzae*

#### Partial cDNA:

A cDNA library was made from *Humicola insolens* strain DSM 1800 mRNA (Kaplan et al. (1979) Biochem.J. 183, 181-184)

according to the method of Okayama and Berg (1982) Mol. Cell. Biol. 2, 161-170. This library was screened by hybridization with radioactively labelled oligonucleotides to filters with immobilized DNA from the recombinants (Gergen et al. (1979) 5 Nucleic Acids Res. 7, 2115-2136). The oligonucleotide probes were made on the basis of aminoacid sequences of tryptic fragments of the purified ~43 kD endoglucanase. A colony was found to hybridize to three different probes (NOR 1251, 2048, and 2050) and was isolated. The sequence showed that the 10 inserted 680 bp cDNA coded for the C-terminal 181 aminoacids of the ~43 kD protein and the 3' nontranslated mRNA. A 237 bp long Pvu I -Xho I fragment from this clone was used to probe a Northern blot (as described in Sambrook et al, op. cit., p. 7.40-7.42 and p. 7.46-7.48.) with H. insolens mRNA and it was 15 shown that the entire ~43 kD mRNA has a length of app. 1100 bp. The same 237 bp fragment was used to probe a genomic library from the same strain.

#### Genomic clone:

A Humicola insolens strain DSM 1800 genomic library was 20 made from total DNA prepared by the method of Yelton (M. M. Yelton et al. (1984) Proc. Natl. Acad. Sci. USA. 81. 1470-1474) and partially digested with Sau 3A. Fragments larger than 4 kb were isolated from an agarose gel and ligated to pBR 322 digested with Bam H1 and dephosphorylated. The ligation 25 products was transformed into E. coli MC1000 (Casadaban and Cohen (1980). J. Mol. Biol., 138, 179-207) made  $r^{-}m^{+}$  by conventional methods. 40.000 recombinants were screened with the 237 bp Pvu I -Xho I partial cDNA fragment described in the paragraph "partial cDNA". 2 colonies that contained the entire 30 ~43 kD endoglucanase sequence were selected and the gene was sequenced by the dideoxy method using the Sequenase<sup>®</sup> kit (United States Biochemical Corporation) according to the manufacturer's instructions. The sequence was identical to the sequence of the full length cDNA gene (see the paragraph "full length cDNA" 35 below) except for one intron in the genomic gene.

The genomic gene was amplified by the PCR method using a Perkin-Elmer/Cetus DNA Amplification System according to the manufacturer's instructions. In the 5' end of the gene the primer NOR 2378 was used. This primer is a 25 mer matching the 5' untranslated end of the gene except for one C to T replacement generating a Bcl I site. In the 3' end of the gene the primer NOR 2389 was used. This primer is a 26 mer of which 21 bases match the 3' untranslated part of the gene and the 5 bases in the 5' end of the primer completes a Sal I site.

10 The Aspergillus expression vector pToC 68 was constructed from plasmid p775 (the construction of which is described in EP 238 023) by insertion of the following linkers

KFN 514: 5'-AGCTGCGGCCGCAGGCCGCGGAGGCCA-3'

KFN 515: 3'-CGCCGGCGTCCGGCGCCTCCGGTTCGA-5'

15 SacII HindIII

EcoRI      NotI      SfiI

KFN 516: 5'-AATTCGCGGCCGCGGCCATGGAGGCC-3'

KFN 519: 3'-GCGCCGGCGCCGGTACCTCCGGTTAA-5'

NcoI

20 The construction of pToC is shown in the appended Fig. 1.

The PCR fragment obtained above was digested with Bcl I and Sal I and inserted into pTOC 68 digested with Bam HI and Xho I. The insert of the resulting plasmid (pCaHj 109) was sequenced and shown to be identical to the original clone.

**25 Full length cDNA:**

First strand cDNA was synthesized from a specific primer within the known sequence (NOR 2153), and second strand synthesis was made by the method described by Gubler and Hoffman (1983) GENE 25, 263-269. The sequence of the genomic gene made it possible to design a PCR primer to catch the 5' part of the mRNA and at the same time introduce a Bam HI site right in front of the ATG start codon (NOR 2334). By using this primer at the 5' end and NOR 2153 again at the 3' end PCR was

performed on the double stranded cDNA product. The full length coding part of the PCR-cDNA was then constructed by cloning the 5' Bam HI - Pvu I fragment from the PCR reaction together with the 3' Pvu I - Eco O109, filled out with Klenow polymerase to make it blunt ended, into Bam HI - Nru I cut Aspergillus expression vector pToC 68 (Fig. 1), and the sequence of the inserted DNA was checked (pSX 320) (cf. Fig. 2). The sequence of the full length cDNA is shown in the appended Sequence Listing ID#1.

#### 10 Oligonucleotide primers used:

NOR 1251: 5'- AAYGCGACAAAYCC -3'

NOR 2048: 5'- AACGAYGAYGGNAAYTTCCC -3'

NOR 2050: 5'- AAYGAYTGGTACCAAYCARTG -3'

NOR 2153: 5'- GCGCCAGTAGCAGCCGGGCTTGAGGG -3'

15 NOR 2334: 5'- ACGTCTCAACTCGGATCCAAGATGCGTT -3'

Bam HI

NOR 2378: 5'- CTCAACTCTGATCAAGATGCGTTCC -3'

Bcl I

NOR 2389: 5'- TGTCCGACCAGTAAGGCCCTCAAGCTG -3'

20 Sal I

#### Nomenclature:

Y: Pyrimidine (C+T)

R: Purine (A+G)

N: All four bases

25 **Enhanced:** Changes or insertions relative to original sequence.

Underlined: Restriction site introduced by PCR.

#### Expression of the ~43 kD endoglucanase:

The plasmid pSX 320 was transformed into Aspergillus oryzae A1560-T40, a protease deficient derivative of A. oryzae IFO 4177, using selection on acetamide by cotransformation with pToC 90 harboring the amdS gene from A. nidulans as a 2.7 kb Xba I fragment (Corrick et al. (1987), GENE 53, 63-71) on a pUC 19 vector (Yannisch-Perron et al. (1985), GENE 33, 103-119).

Transformation was performed as described in the published EP patent application No. 238 023. A number of transformants were screened for co-expression of ~43 kD endoglucanase. Transformants were evaluated by SDS-PAGE (p.3) and CMC 5 endoglucanase activity.

The plasmid containing the genomic gene (pCaHj 109) was transformed into Aspergillus oryzae A1560-T40 by the same procedure. Evaluation of the transformants showed that the level of expression was similar to that of the cDNA 10 transformants.

The purified ~43 kD endoglucanase was analysed for its N-terminal sequence and carbohydrate content. The N-terminal amino acid sequence was shown to be identical to that of the HPLC purified ~43 kD endoglucanase. The carbohydrate content 15 differs from that of the HPLC purified ~43 kD enzyme in that the recombinant enzyme contains 10 +/- 8 galactose sugars per mol rather than glucose.

### Example 3

#### Cloning of *Fusarium oxysporum* ~43 kD endoglucanase

20 To isolate the Fusarium homologue to the Humicola ~43 kD cellulase a fragment was first obtained by PCR (as described by Lee et al., Science 239, 1988, pp. 1288-1291) and cloned. This product was then sequenced and primers to be used as library probes and for PCR amplification were constructed. 25 These oligonucleotides were used to isolate the corresponding clone from a cDNA library.

PCR was used to isolate partial length cDNA and genomic fragments of the 43 kD homologue. Seven different combinations of highly degenerate oligonucleotides (see table below) were 30 used in PCR reactions with either cDNA or genomic DNA as templates. Two separate sets of PCR conditions were used for each oligonucleotide pair; the first set was designed to make very little product but with very high specificity. Various factors ensured specificity in this set of 28 cycles: The 35 annealing temperature of 65°C was very high for these

oligonucleotides; the time at annealing temperature was set for only 30 seconds; 20 picomoles of each degenerate primer mixture was used per 100  $\mu$ l reaction. The oligonucleotides used contained only the degenerate region without a "tail" to be used for cloning; 1 unit of Amplitaq polymerase (Perkin-Elmer Cetus) was used per 100  $\mu$ l reaction; and EDTA was added to reaction tubes at the end of the final 10 minute 72°C incubation to prevent extension from mismatched primers at cooler temperatures following the PCR cycles. Products of the first set of cycles would not be expected to be visible by ethidium bromide staining in agarose gel electrophoresis due to the low efficiency of amplification required to ensure high specificity. The second set of amplifications was, however, designed to efficiently amplify products from the first set. Factors ensuring this include: Lowering the annealing temperature to 55°C; Lengthening the time of annealing to 1 minute; Increasing the amount of oligonucleotides to 100 picomoles of each mixture per 100  $\mu$ l reaction; Utilizing a different set of oligonucleotides which include a "Prime" cloning tail along with the degenerate portion (increasing the melting temperature dramatically) and by using 2.5 units of Amplitaq polymerase per 100  $\mu$ l reaction.

PCR reactions were set up as recommended by Perkin Elmer Cetus. A master mix was made for each of 2 DNA sources, genomic and cDNA. This was comprised of 1X PCR buffer (10 mM Tris/HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.01% gelatin, Perkin Elmer Cetus), 0.2 mM deoxynucleotides (Ultrapure dNTP 100 mM solution, Pharmacia), 1 unit Amplitaq polymerase (Perkin Elmer Cetus) and 0.5  $\mu$ g genomic DNA or 50 ng cDNA per 100  $\mu$ l reaction mixture volume, and deionized water to bring volume up to 98  $\mu$ l per 100  $\mu$ l reaction. To labeled 0.5 tubes (Eppendorf) were added 20 picomoles (1  $\mu$ l of a 20 picomole/ $\mu$ l concentration) of each oligonucleotide mixture (see table below). These were placed in a Perkin Elmer Cetus thermocycler at 75°C along with the master mixes and light mineral oil also in 0.5 ml tubes. Ninety eight microliters of the appropriate master mix and 55  $\mu$ l light mineral oil were added to each tube with

oligonucleotides. The reactions were then started in a step-cycle file (see chart below for parameters). At the end of the final 72°C incubation, 50  $\mu$ l of a 10 mM EDTA pH 8.0 solution was added to each tube and incubated for a further 5 minutes at 5 72°C.

Table of oligonucleotide pairs used in 43 kD homologue PCR:

|    | reaction |         | oligos for first set degenerate only | oligos for second set degenerate with "prime" | expected size in base pairs |
|----|----------|---------|--------------------------------------|---|-----------------------------|
|    | 10 cDNA  | genomic |                                      |   |                             |
|    | 1        | 11      | ZC3485 vs ZC3558                     | ZC3486 vs ZC3559                              | 288                         |
| 15 | 2        | 12      | ZC3485 vs ZC3560                     | ZC3486 vs ZC3561                              | 510                         |
|    | 3        | 13      | ZC3485 vs ZC3264                     | ZC3486 vs ZC3254                              | 756                         |
|    | 4        | 14      | ZC3556 vs ZC3560                     | ZC3557 vs ZC3561                              | 159                         |
| 20 | 5        | 15      | ZC3556 vs ZC3264                     | ZC3557 vs ZC3254                              | 405                         |
|    | 6        | 16      | ZC3556 vs ZC3465                     | ZC3557 vs ZC3466                              | 405                         |
| 25 | 7        | 17      | ZC3485 vs ZC3465                     | ZC3486 vs ZC3466                              | 756                         |

Note: See oligonucleotide table for oligonucleotide sequences

Conditions for PCR step-cycle file were:

| SET 1: |      |      |        | SET 2: |      |        |  |
|--------|------|------|--------|--------|------|--------|--|
| 30     |      | 94°C | 1 min  |        | 94°C | 1 min  |  |
|        | 28 X | 65°C | 30 sec | 28 X   | 55°C | 1 min  |  |
|        |      | 72°C | 2 min  |        | 72°C | 2 min  |  |
|        |      | 72°C | 10 min |        | 72°C | 10 min |  |

Following the first set of PCR cycles, DNA was purified from the reaction mixtures by isopropyl alcohol precipitation for use in the second set of cycles. Most of the light mineral oil was removed from the top of each sample before transferring



the sample to a new labeled tube. Each tube was then extracted with an equal volume PC1 (49% phenol: 49% chloroform: 2% isoamyl alcohol) and then with an equal volume of chloroform. DNA was then precipitated from the reactions by adding: 75  $\mu$ l  
5 7.5 M ammonium acetate, 1  $\mu$ l glycogen and 226  $\mu$ l isopropyl alcohol. Pellets were resuspended in 20  $\mu$ l deionized water. Two microliters of each resuspension were placed into labeled tubes for the second round of PCR amplifications along with 100 picomoles (5 $\mu$ l of a 20 picomole/ $\mu$ l concentration ) of each new  
10 primer mixture (see table above). A master mix was made as described above except for excluding the DNA and compensating for increased oligonucleotide and DNA volumes in the reaction tubes. Reactions and cycles were set up as described above (see table above).

15 To isolate full length cDNA clones of the 43 kD homologue a library of 1,100,000 clones was plated out onto 150 mm LB plates with 100  $\mu$ g/ml ampicillin. One hundred thousand clones were plated out from glycerol stocks onto each of 10 plates and 20,000 clones were plated out on each of 5 plates.  
20 Lifts were taken in duplicate as described above. Prehybridization, hybridization and washing were also carried out as described above. Two end labeled 42-mer oligonucleotides, ZC3709 and ZC3710, were used in the hybridization. Filters were washed one time for 20 minutes with TMACL at 77°C. Twenty two  
25 spots showing up on duplicate filters were found. Corresponding areas on the plates were picked with the large end of a pipet into 1 ml of 1 X PCR buffer. These were PCRed with 2 sets of oligonucleotides. One set contained the two 43 kD specific oligonucleotides used as hybridization probes and the other  
30 contained one 43 kD specific oligonucleotide, ZC3709, and one vector specific oligonucleotide, ZC3634. PCR was conducted as before by Perkin Elmer Cetus directions. Twenty picomoles of each primer and 5  $\mu$ l of the cell suspension were used in each reaction of 50  $\mu$ l. After an initial 1 minute 30 second  
35 denaturation at 94°C 30 cycles of 1 minute at 94°C and 2 minutes at 72°C were employed, with a final extension time of 10 minutes at 72°C. Results showed 17 of the 22 to contain the

2 43 kD specific oligonucleotide recognition sites. The other  
5 clones contained one of the 2 sites, ZC3709, but were shown  
by PCR with the vector specific primer to be truncated and not  
long enough to contain the other site. The 9 longest clones  
5 were chosen for single colony isolation through another level  
of screening. Five 10 fold dilutions of each were plated out  
and processed as described above for the first set of lifts.  
All of the nine had signals on autoradiograms of the second  
level of screening. Colonies were fairly congested so a few  
10 separate colonies in the area of the radioactive signal were  
single colony isolated on 150 mm LB plates with 70 µg/ml  
ampicillin. These were tested by PCR for homologues to the 43  
kD cellulase with the oligonucleotides ZC3709 and ZC3710 as  
described for the first level of screening except that colonies  
15 were picked by toothpick into 25 µl of mastermix. Bands of the  
expected size were obtained for 7 of the 9 clones. Cultures of  
these were started in 20 ml of Terrific Broth with 150 µg/ml  
ampicillin. DNA was isolated by alkaline lysis and PEG  
precipitation as above.

## 20 DNA sequence analysis

A freeze-dried culture of Fusarium oxysporum was  
reconstituted with phosphate buffer, spotted 5 times on each of  
5 FOX medium plates (6% yeast extract, 1.5% K<sub>2</sub>HPO<sub>4</sub>, 0.75% MgSO<sub>4</sub>  
7H<sub>2</sub>O, 22.5% glucose, 1.5% agar, pH 5.6) and incubated at 37°C.  
25 After 6 days of incubation the colonies were scraped from the  
plates into 15 ml of 0.001% Tween-80 which appeared as a thick  
and cloudy suspension.

Four 1-liter flasks, each containing 300 ml of liquid  
FOX medium, were inoculated with 2 ml of the spore suspension  
30 and were incubated at 30°C and 240 RPM. On the 4th day of  
incubation, the cultures were filtered through 4 layers of  
sterile gauze and washed with sterile water. The mycelia were  
dried on Whatman filter paper, frozen in liquid nitrogen,  
ground into a fine powder in a cold pestle and added to 75 ml  
35 of fresh lysis buffer (10 mM Tris-Cl 7.4, 1% SDS, 50 mM EDTA,  
100 µL DEPC). The thoroughly mixed suspension was incubated in

a 65°C waterbath for 1 hour and then spun for 10 minutes at 4000 RPM and 5°C in a bench-top centrifuge. The supernatant was decanted and EtOH precipitated. After 1 hour on ice the solution was spun at 19,000 RPM for 20 minutes. The supernatant was decanted and isopropanol precipitated. Following centrifugation at 10,000 RPM for 10 minutes, the supernatant was decanted and the pellets allowed to dry.

One milliliter of TER solution (TE 7.4 + 10 uL 2000 unites RNase) was added to each tube, which were stored at 4°C for two days. The tubes were pooled and placed in a 65°C waterbath for 30 minutes to suspend non-dissolved DNA. The solution was extracted twice with phenol/CHCl<sub>3</sub>/isoamyl alcohol, twice with CHCl<sub>3</sub>/isoamyl alcohol and then ethanol precipitated. The pellet was allowed to settle and the EtOH removed. 70% EtOH was added and the DNA stored overnight at -20°C. After decanting and drying, 1 ml of TER was added and the DNA dissolved by incubating the tubes at 65°C for 1 hour. The preparation yielded 1.5 mg of genomic DNA.

Oligonucleotides for 43 kD homologue PCR:

20 ZC3485 TGG GA(C/T) TG(C/T) TG(C/T) AA(A/G) CC  
 ZC3486 AGG GAG ACC GGA ATT CTG GGA (C/T)TG (C/T)TG (C/T)  
 AA(A/G) CC  
 ZC3556 CC(A/C/G/T) GG(A/C/G/T) GG(A/C/G/T) GG(A/C/G/T)  
 GT(A/C/G/T) GG  
 25 ZC3557 AGG GAG ACC GGA ATT CCC (A/C/G/T)GG (A/C/G/T)GG  
 (A/C/G/T)GG (A/C/G/T)GT (A/C/G/T)GG  
 ZC3558 AC(A/C/G/T) A(C/T)C AT(A/C/G/T) (G/T)T/C/T) TT(A/C/G/T)  
 CC  
 ZC3559 GAC AGA GCA CAG AAT TCA C(A/C/G/T)A (C/T)CA  
 30 T(A/C/G/T)(G/T) T(C/T)T T(A/C/G/T)C C  
 ZC3560 (A/C/G/T)GG (A/G)TT (A/G)TC (A/C/G/T)GC  
 (A/C/G/T)(G/T)(C/T) (C/T)T(C/T) (A/G)AA CCA  
 ZC3561 GAC AGA GCA CAG AAT TC(A/C/G/T) GG(A/G) TT(A/G)  
 TC(A/C/G/T) GC(A/C/G/T) (G/T)(C/T)(C/T) T(C/T)(A/G)  
 35 AAC CA

Oligonucleotides for 43 kD homologue cloning:

ZC3709 GGG GTA GCT ATC ACA TTC GCT TCG GGA GGA GAT ACC GCC  
GTA

ZC3710 CTT CTT GCT CTT GGA GCG GAA AGG CTG CTG TCA ACG CCC  
5 CTG

pYCDE8' vector oligonucleotides:

ZC3635 TGT ACG CAT GTA ACA TTA CYC 1 terminator  
ZC3634 CTG CAC AAT ATT TCA AGC ADH 1 promoter

Example 410 Colour clarification test

The Humicola ~43 kD endoglucanase (a mixture of 30 purification runs) was compared in a colour clarification test with the H. insolens cellulase preparation described in US 4,435,307, Example 6.

15 Old worn black cotton swatches are used as the test material. The clarification test is made in a Terg-O-tometer making three repeated washes. Between each wash the swatches are dried overnight.

Conditions:

20 2 g/l of liquid detergent at 40°C for 30 min. and a water hardness of 9°dH. The swatch size is 10x15 cm, and there are two swatches in each beaker.

The composition of the detergent was as follows:

10% anionic surfactant (Nansa 1169/p)  
25 15% non-ionic surfactant (Berol 160)  
10% ethanol  
5% triethanol amine  
60% water

pH adjusted to 8.0 with HCl.

30 Dosage:

The two enzymes are dosed in 63 and 125 CMC-endoase units/l.

**Results:**

The results were evaluated by a panel of 22 persons who rated the swatches on a scale from 1 to 7 points. The higher the score, the more colour clarification obtained.

|    |                                     |               |              |                        |
|----|-------------------------------------|---------------|--------------|------------------------|
| 5  | Enzyme                              | CMC-endoase/l | Protein mg/l | PSU*                   |
| 10 | No enzyme                           |               |              | 1.4 ± 1.0              |
| 15 | H. insolens<br>cellulase<br>mixture | 63<br>125     | 14<br>28     | 5.8 ± 1.0<br>6.1 ± 1.0 |
| 20 | Invention                           | 63<br>125     | 0.4<br>0.8   | 4.6 ± 0.9<br>6.2 ± 0.8 |

\* PSU = Panel Score Units

The ~43 kD endoglucanase is shown to have an about 30 times better performance than the prior art H. insolens cellulase mixture and an about 6 times better performance than the cellulase preparation according to WO 89/09259.

**Example 5**

**Stability of the Humicola ~43 kD endoglucanase in the presence of proteases**

30 The storage stability of the ~43 kD endoglucanase in liquid detergent in the presence of different proteases was determined under the following conditions:

Enzymes

- ~43 kD endoglucanase of the invention  
Glu/Asp specific B. licheniformis serine protease  
Trypsin-like Fusarium sp. DSM 2672 protease  
5 B. lentus serine protease  
Subtilisin Novo

Detergent

US commercial liquid detergent not containing any opacifier, perfume or enzymes (apart from those added in the 10 experiment). +/- 1% (w/w) boric acid as enzyme stabiliser.

Dosage

|                |                        |
|----------------|------------------------|
| Endoglucanase: | 12 CMCU/g of detergent |
| Proteases:     | 0.2 mg/g of detergent  |

Incubation

- 15 7 days at 35°C

Residual activity

The residual activity of the endoglucanase after 7 days of incubation with the respective proteases was determined in terms of its CMCase activity (CMCU).

- 20 The CMCase activity was determined as follows:

A substrate solution of 30 g/l CMC (Hercules 7 LFD) in deionized water was prepared. The enzyme sample to be determined was dissolved in 0.01 M phosphate buffer, pH 7.5. 1.0 ml of the enzyme solution and 2.0 ml of a 0.1 M phosphate 25 buffer, pH 7.5, were mixed in a test tube, and an enzyme reaction was initiated by adding 1.0 ml of the substrate solution to the test tube. The mixture was incubated at 40°C for 20 minutes, after which the reaction was stopped by adding 2.0 ml of 0.125 M trisodium phosphate.12H<sub>2</sub>O. A blind sample was 30 prepared without incubation.

2.0 ml of a ferricyanide solution (1.60 g of potassium ferricyanide and 14.0 g of trisodium phosphate.12H<sub>2</sub>O in 1 l of deionized water) was added to a test sample as well as to a blind immediately followed by immersion in boiling water and 5 incubation for 10 minutes. After incubation, the samples were cooled with tap water. The absorbance at 420 nm was measured, and a standard curve was prepared with glucose solution.

One CMCase unit (CMCU) is defined as the amount of enzyme which, under the conditions specified above, forms an 10 amount of reducing carbohydrates corresponding to 1  $\mu$ mol of glucose per minute.

### Results

The storage stability of the endoglucanase of the invention was determined as its residual activity (in CMCU%) 15 under the conditions indicated above.

|    | Protease                | Residual Activity (%) |              |
|----|-------------------------|-----------------------|--------------|
|    |                         | + boric acid          | - boric acid |
| 20 | Glu/Asp specific        | 105                   | 93           |
|    | Trypsin-like            | 77                    | 63           |
|    | <u>B. lentus</u> serine | 57                    | 24           |
|    | Subtilisin Novo         | 63                    | 55           |

These results indicate that the storage stability in liquid 25 detergent of the endoglucanase of the invention is improved when a protease with a higher degree of specificity than Savinase is included in the detergent composition.

### Example 6

Use of Humicola ~43 kD endoglucanase to provide a localized 30 variation in colour of denim fabric

Denim jeans were subjected to treatment with the ~43 kD endoglucanase in a 12 kg "Wascator" FL120 wash extractor with

a view to imparting a localized variation in the surface colour of the jeans approximating a "stonewashed" appearance.

Four pairs of jeans were used per machine load. The experimental conditions were as follows.

#### 5 Desizing

- 40 l water
- 100 ml B. amyloliquefaciens amylase\*, 120 L
- 70 g  $\text{KH}_2\text{PO}_4$
- 30 g  $\text{Na}_2\text{HPO}_4$
- 10 55°C
- 10 minutes
- pH 6.8

\*available from Novo Nordisk A/S.

The desizing process was followed by draining.

#### 15 Abrasion

- 40 l water
- 120 g H. insolens cellulase mixture or
- x g ~43 kD endoglucanase
- 70 g  $\text{KH}_2\text{PO}_4$
- 20 30 g  $\text{Na}_2\text{HPO}_4$
- 55°C
- 75 minutes
- pH 6.6

The abrasion process was followed by draining, rinsing, after-  
25 washing and rinsing.

The results were evaluated by judging the visual appearance of the jeans.

Different dosages of ~43 kD endoglucanase were used to obtain an abrasion level which was equivalent to that obtained  
30 with 120 g H. insolens cellulase mixture. Such an equivalent level was obtained with 1.0-1.25 g of ~43 kD endoglucanase. Measurements of the tear strength of the treated garments showed no significant difference between the two enzyme treatments.



Example 7Use of *Humicola* ~43 kD endoglucanase to remove fuzz from fabric surface

Woven, 100% cotton fabric was treated with the ~43 kD endoglucanase in a 12 kg "Wascator" FL120 wash extractor with a view to investigating the ability of the enzyme to impart a greater degree of softness to new fabric.

The experimental conditions were as follows.

Fabric

- 10 Woven, 100% cotton fabric obtained from Nordisk Textil, bleached (NT2116-b) or unbleached (NT2116-ub). 400 g of fabric were used per machine load.

Desizing

- 40 l water  
15 200 ml *B. amyloliquefaciens* amylase, 120 L  
60 g  $\text{KH}_2\text{PO}_4$   
20 g  $\text{Na}_2\text{HPO}_4$   
60°C  
10 minutes  
20 pH 6.4

The desizing process was followed by draining.

Main wash

- 40 l water  
0-600 g *H. insolens* cellulase mixture or  
25 x g ~43 kD endoglucanase  
60 g  $\text{KH}_2\text{PO}_4$   
40 g  $\text{Na}_2\text{HPO}_4$   
60°C  
60 minutes  
30 pH 6.7

The abrasion step was followed by draining.

Afterwash

40 l water  
40 g  $\text{Na}_2\text{CO}_3$   
10 g Berol 08  
5 80°C  
15 minutes  
pH 10.1

The afterwash was followed rinsing.

Three different concentrations of the ~43 kD  
10 endoglucanase were added in the main wash.

The weight loss of the fabric samples was measured before and after treatment. The weight loss is expressed in % and is related to the desized fabric.

Fabric thickness was measured by means of a thickness  
15 measurer L&W, type 22/1. 2 swatches of the fabric (10 x 6 cm) were measured, and 5 measurements in  $\mu\text{m}$  were recorded for each swatch. The swatch was measured at a pressure of 98.07 kPa. The retained thickness is expressed in % in relation to the desized fabric.

20 Fabric strength was measured by means of a tearing tester (Elmendorf 09). 6 swatches (10 x 6 cm) were cut in the warp direction and 6 swatches (10 x 6 cm) in the weft direction. The tear strength was measured in mN in accordance with ASTM D 1424. The fabric strength of the enzyme-treated  
25 fabric is expressed in % in relation to the desized fabric.

Fabric stiffness was measured by means of a King Fabric Stiffness Tester. 4 swatches (10 x 20 cm; 10 cm in the warp direction) are cut from the fabric, and each swatch is folded back to back (10 x 10 cm) and placed on a table provided with  
30 an open ring in the middle. A piston pushes the fabric through the ring using a certain power expressed in grammes. The determination is made according to the ASTM D 4032 Circular Bend Test Method. Retained fabric stiffness is expressed in % in relation to the desized fabric.

Judged by these criteria, the use of the ~43 kD endoglucanase has an effect on weight loss as well as fabric thickness. The weight loss is considered to correlate with the improvement of fabric "handle" and appearance. Positive effects were observed on fabric thickness which are assumed to correlate with fuzz removed from the fabric surface.

#### Example 8

#### Use of *Humicola* ~43 kD endoglucanase for the treatment of paper pulp

10 The ~43 kD endoglucanase was used for the treatment of several types of paper pulp with a view to investigating the effect of the enzyme on pulp drainage.

The experimental conditions were as follows.

#### Pulps

- 15 1. Waste paper mixture: composed of 33% newsprint, 33% magazines and 33% computer paper. With or without deinking chemicals (WPC or WP, respectively)
2. Recycled cardboard containers (RCC).
3. Bleached kraft: made from pine (BK).
- 20 4. Unbleached thermomechanical: made from fir (TMP).

#### Determination of cellulase activity (CEVU)

A substrate solution containing 33.3 g/l CMC (Hercules 7 LFD) in Tris-buffer, pH 9.0, is prepared. The enzyme sample to be determined is dissolved in the same buffer. 10 ml  
25 substrate solution and 0.5 ml enzyme solution are mixed and transferred to a viscosimeter (Haake VT 181, NV sensor, 181 rpm) thermostated at 40°C. One Cellulase Viscosity Unit (CEVU) is defined in Novo Nordisk Analytical Method No. AF 253 (available from Novo Nordisk).

30

#### Determination of pulp drainage (Schopper-Riegler)

The Schopper-Riegler number (SR) is determined according to ISO standard 5267 (part 1) on a homogenous pulp with a

consistency of 2 g/l. A known volume of pulp is allowed to drain through a metal sieve into a funnel. The funnel is provided with an axial hole and a side hole. The volume of filtrate that passes through the side hole is measured in a 5 vessel graduated in Schopper-Riegler units.

#### Enzymatic treatment

A preparation of the ~43 kD endoglucanase was diluted to 7 CEVU/ml and added to each of the pulps indicated above (50 g DS, consistency 3%). The enzyme dose was 2400 CEVU/kg dry 10 pulp. The enzymatic treatment was conducted at a pH of 7.5 and at 40°C with gentle stirring for 60 minutes. A sample was taken after 30 minutes to monitor the progression of the reaction. After 60 minutes, the pulp was diluted to a consistency of 0.5% with cold water (+4°C) in order to stop the reaction.

15 Drainage of the wet pulp was determined as described above and assigned Schopper-Riegler (SR) values. The drainage time (DT) under vacuum was also determined.

The results are summarized in the following table.

|    |   | Waste paper + chemicals |        |
|----|---|-------------------------|--------|
|    |   | Control                 | Enzyme |
| 20 | SR (3%)                                   | 61                      | 55     |
|    | Drainage time (s)<br>150 g/m <sup>2</sup> | 18.2                    | 17     |
| 25 | Mass g/m <sup>2</sup>                     | 65.6                    | 66.4   |
|    | Vol cm <sup>3</sup> /g                    | 1.65                    | 1.66   |
| 30 | Breaking Length, m                        | 3650                    | 3970   |
|    | Burst Index                               | 2.19                    | 2.47   |

36

|    |   | Waste paper |        |
|----|---|-------------|--------|
|    |   | Control     | Enzyme |
| 5  | SR (3%)                                   | 59          | 51     |
|    | Drainage time (s)<br>150 g/m <sup>2</sup> | 18.2        | 12.7   |
| 10 | Mass g/m <sup>2</sup>                     | 68.0        | 67.9   |
|    | Vol cm <sup>3</sup> /g                    | 1.68        | 1.64   |
| 15 | Breaking Length, m                        | 3810        | 3790   |
|    | Burst Index                               | 2.25        | 2.33   |

|    |   | Recycled Cardboard Containers |        |
|----|---|-------------------------------|--------|
|    |   | Control                       | Enzyme |
| 20 |   |                               |        |
| 25 | SR (3%)                                   | 45                            | 33     |
|    | Drainage time (s)<br>150 g/m <sup>2</sup> | 6.8                           | 5.3    |
| 30 | Mass g/m <sup>2</sup>                     | 70.2                          | 67.3   |
|    | Vol cm <sup>3</sup> /g                    | 1.91                          | 1.99   |
|    | Breaking Length, m                        | 3640                          | 3530   |
| 35 | Burst Index                               | 2.25                          | 2.22   |

|   | Kraft   |        |
|---|---------|--------|
|   | Control | Enzyme |
| 5 SR (3%)                                 | 42      | 31     |
| Drainage time (s)<br>150 g/m <sup>2</sup> | 10.7    | 6      |
| 10 Mass g/m <sup>2</sup>                  | 67.5    | 69,1   |
| Vol cm <sup>3</sup> /g                    | 1.44    | 1.42   |
| 15 Breaking Length, m                     | 7010    | 7190   |
| Burst Index                               | 5.14    | 4.96   |

|  | TMP     |        |
|--|---------|--------|
|  | Control | Enzyme |
| 20 SR (3%)                                   | 68      | 60     |
| 25 Drainage time (s)<br>150 g/m <sup>2</sup> | 13.8    | 11.3   |
| Mass g/m <sup>2</sup>                        | 68.7    | 70.2   |
| 30 Vol cm <sup>3</sup> /g                    | 2.13    | 2.04   |
| Breaking Length, m                           | 3630    | 3620   |
| 35 Burst Index                               | 1.95    | 1.91   |

Tabel 3: Results of the drainage and strength measurements.

Control experiments. Same conditions as the enzyme treatment,

It appears from the table that the ~43 kD endoglucanase treatment causes a significant decrease in SR values and significantly improves drainage of pulps used in papermaking.

Paper sheets were made from the various pulps on a Rapid Köthen device and measured for strength according to different parameters (including breaking length). No decrease in strength properties due to enzyme action was observed.

## CLAIMS

1. A cellulase preparation consisting essentially of a homogenous endoglucanase component which is immunoreactive with an antibody raised against a highly purified ~43 kD endoglucanase derived from Humicola insolens, DSM 1800, or which is homologous to said ~43 kD endoglucanase.

2. A cellulase preparation according to claim 1, wherein the endoglucanase component has an endoglucanase activity of at least 50 CMC-endoase units/mg of protein.

10 3. A cellulase preparation according to claim 2, wherein the endoglucanase component has an endoglucanase activity of at least 60 CMC-endoase units/mg of total protein, in particular at least 90 CMC-endoase units/mg of total protein, and preferably at least 100 CMC-endoase units/mg of total protein.

15 4. A cellulase preparation according to claim 1, wherein the endoglucanase component has essentially no cello-biohydrolase activity.

5. A cellulase preparation according to any of claims 1-4, wherein the endoglucanase component has an isoelectric 20 point of about 5.1.

6. An enzyme exhibiting endoglucanase activity, which enzyme has the amino acid sequence shown in the appended Sequence Listing ID#2, or a homologue thereof exhibiting endoglucanase activity.

25 7. An endoglucanase enzyme according to claim 6 which is producible by a species of Humicola, e.g. Humicola insolens.

8. An enzyme exhibiting endoglucanase activity, which enzyme has the amino acid sequence shown in the appended Sequence Listing ID#4, or a homologue thereof exhibiting 30 endoglucanase activity.

9. An endoglucanase enzyme according to claim 8 which is producible by a species of Fusarium, e.g. Fusarium oxysporum.

10. A DNA construct comprising a DNA sequence encoding 35 an endoglucanase enzyme as claimed in any of claims 6-9.



11. A DNA construct according to claim 10, wherein the DNA sequence is as shown in the appended Sequence Listings ID#1 or ID#3 or a modification thereof.

12. An expression vector which carries an inserted DNA sequence according to claim 10 or 11.

13. A cell which is transformed with a DNA construct according to claim 10 or 11 or with an expression vector according to claim 12.

14. A cell according to claim 13 which is a fungal cell, e.g. belonging to a strain of Trichoderma or Aspergillus, in particular Aspergillus oryzae or Aspergillus niger, or a yeast cell, e.g. belonging to a strain of Hansenula or Saccharomyces, e.g. Saccharomyces cerevisiae.

15. A process for producing an endoglucanase enzyme as defined in any of claims 6-9, the process comprising culturing a cell according to claim 13 or 14 in a suitable culture medium under conditions permitting the expression of the endoglucanase enzyme, and recovering the endoglucanase enzyme from the culture.

16. A detergent additive containing a cellulase preparation according to any of claims 1-5 or an endoglucanase enzyme according to any of claims 6-9, preferably in the form of a non-dusting granulate, stabilized liquid or protected enzyme.

17. A detergent additive according to claim 16 which contains 1-500, preferably 5-250, most preferably 10-100, mg of enzyme protein per gram of the additive.

18. A detergent additive according to claim 16 which additionally comprises another enzyme such as a protease, lipase, peroxidase and/or amylase.

19. A detergent additive according to claim 18, wherein the protease is one which has a higher degree of specificity than Bacillus lentus serine protease.

20. A detergent additive according to claim 19, wherein the protease is subtilisin Novo or a variant thereof, a protease derivable from Nocardia dassonvillei NRRL 18133, a serine protease specific for glutamic and aspartic acid,

producible by Bacillus licheniformis, or a trypsin-like protease producible by Fusarium sp. DSM 2672.

21. A detergent composition comprising a cellulase preparation according to any of claims 1-5 or an endoglucanase 5 enzyme according to any of claims 6-9.

22. A detergent composition according to claim 21, which additionally comprises another enzyme such as a protease, lipase, peroxidase and/or amylase.

23. A detergent composition according to claim 22, 10 wherein the protease is one which has a higher degree of specificity than Bacillus lentus serine protease.

24. A detergent composition according to claim 23, wherein the protease is subtilisin Novo or a variant thereof, a protease derivable from Nocardia dassonvillei NRRL 18133, a 15 serine protease specific for glutamic and aspartic acid, producible by Bacillus licheniformis, or a trypsin-like protease producible by Fusarium sp. DSM 2672.

25. A detergent composition according to claim 21, wherein the cellulase preparation or endoglucanase enzyme is 20 present in a concentration corresponding to 0.01-100, preferably 0.05-60, and most preferably 0.1-20, mg of enzyme protein per liter washing solution.

26. A detergent composition comprising a detergent additive according to any of claims 16-20.

25 27. A method of reducing the rate at which cellulose-containing fabrics become harsh or of reducing the harshness of cellulose-containing fabrics, the method comprising treating cellulose-containing fabrics with a cellulase preparation according to any of claims 1-5 or an endoglucanase enzyme 30 according to any of claims 6-9.

28. A method of providing colour clarification of coloured cellulose-containing fabrics, the method comprising treating coloured cotton-containing fabrics with a cellulase preparation according to any of claims 1-5 or an endoglucanase 35 enzyme according to any of claims 6-9.

29. A method of providing a localized variation in colour of coloured cellulose-containing fabrics, the method

comprising treating coloured cotton-containing fabrics with a cellulase preparation according to any of claims 1-5 or an endoglucanase enzyme according to any of claims 6-9.

30. A method according to any of claims 27, 28 or 29,  
5 wherein the treatment of the fabrics with the cellulase preparation is carried out during soaking, washing or rinsing of the fabrics.

31. A method of improving the drainage properties of pulp, the method comprising treating paper pulp with a  
10 cellulase preparation according to any of claims 1-5 or an endoglucanase enzyme according to any of claims 6-9.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- 5 (i) APPLICANT: NOVO NORDISK A/S, N N
- (ii) TITLE OF INVENTION: A Cellulase Preparation
- 10 (iii) NUMBER OF SEQUENCES: 4
- (iv) CORRESPONDENCE ADDRESS:
- (A) ADDRESSEE: NOVO NORDISK A/S, Patent Department
- 15 (B) STREET: Novo Alle
- (C) CITY: Bagsvaerd
- (E) COUNTRY: DENMARK
- (F) ZIP: DK-2880
- (v) COMPUTER READABLE FORM:
- 20 (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- 25 (vi) CURRENT APPLICATION DATA:
- (A) APPLICATION NUMBER:
- (B) FILING DATE:
- (C) CLASSIFICATION:
- 30 (viii) ATTORNEY/AGENT INFORMATION:
- (A) NAME: Thaloe-Madsen, Birgit
- (ix) TELECOMMUNICATION INFORMATION:
- 35 (A) TELEPHONE: +45 4444 8888
- (B) TELEFAX: +45 4449 3256
- (C) TELEX: 37304

## (2) INFORMATION FOR SEQ ID NO:1:

- 40 (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 1060 base pairs
- (B) TYPE: nucleic acid
- 45 (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- 50 (vi) ORIGINAL SOURCE:
- (A) ORGANISM: Humicola insolens
- (B) STRAIN: DSM 1800

44

## (ix) FEATURE:

(A) NAME/KEY: mat\_peptide  
(B) LOCATION: 73..927

5

## (ix) FEATURE:

(A) NAME/KEY: sig\_peptide  
(B) LOCATION: 10..72

10

## (ix) FEATURE:

(A) NAME/KEY: CDS  
(B) LOCATION: 10..927

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

|    |   |     |
|----|---|-----|
| 15 | GGATCCAAG ATG CGT TCC TCC CCC CTC CTC CCG TCC GCC GTT GTG GCC   | 48  |
|    | Met Arg Ser Ser Pro Leu Leu Pro Ser Ala Val Val Ala             |     |
|    | -21 -20 -15 -10   |     |
| 20 | GCC CTG CCG GTG TTG GCC CTT GCC GCT GAT GGC AGG TCC ACC CGC TAC | 96  |
|    | Ala Leu Pro Val Leu Ala Leu Ala Ala Asp Gly Arg Ser Thr Arg Tyr |     |
|    | -5 1 5  |     |
|    | TGG GAC TGC TGC AAG CCT TCG TGC GGC TGG GCC AAG AAG GCT CCC GTG | 144 |
| 25 | Trp Asp Cys Cys Lys Pro Ser Cys Gly Trp Ala Lys Lys Ala Pro Val |     |
|    | 10 15 20  |     |
|    | AAC CAG CCT GTC TTT TCC TGC AAC GCC AAC TTC CAG CGT ATC ACG GAC | 192 |
| 30 | Asn Gln Pro Val Phe Ser Cys Asn Ala Asn Phe Gln Arg Ile Thr Asp |     |
|    | 25 30 35 40   |     |
|    | TTC GAC GCC AAG TCC GGC TGC GAG CCG GGC GGT GTC GCC TAC TCG TGC | 240 |
|    | Phe Asp Ala Lys Ser Gly Cys Glu Pro Gly Gly Val Ala Tyr Ser Cys |     |
|    | 45 50 55  |     |
| 35 | GCC GAC CAG ACC CCA TGG GCT GTG AAC GAC GAC TTC GCG CTC GGT TTT | 288 |
|    | Ala Asp Gln Thr Pro Trp Ala Val Asn Asp Asp Phe Ala Leu Gly Phe |     |
|    | 60 65 70  |     |
| 40 | GCT GCC ACC TCT ATT GCC GGC AGC AAT GAG GCG GGC TGG TGC TGC GCC | 336 |
|    | Ala Ala Thr Ser Ile Ala Gly Ser Asn Glu Ala Gly Trp Cys Cys Ala |     |
|    | 75 80 85  |     |
|    | TGC TAC GAG CTC ACC TTC ACA TCC GGT CCT GTT GCT GGC AAG AAG ATG | 384 |
| 45 | Cys Tyr Glu Leu Thr Phe Thr Ser Gly Pro Val Ala Gly Lys Lys Met |     |
|    | 90 95 100   |     |
|    | GTC GTC CAG TCC ACC AGC ACT GGC GGT GAT CTT GGC AGC AAC CAC TTC | 432 |
|    | Val Val Gln Ser Thr Ser Thr Gly Gly Asp Leu Gly Ser Asn His Phe |     |
| 50 | 105 110 115 120   |     |
|    | GAT CTC AAC ATC CCC GGC GGC GGC GTC GGC ATC TTC GAC GGA TGC ACT | 480 |
|    | Asp Leu Asn Ile Pro Gly Gly Gly Val Gly Ile Phe Asp Gly Cys Thr |     |
|    | 125 130 135   |     |

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|    |  |            |                   |                   |   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |     |      |
|----|--|------------|-------------------|-------------------|---|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-----|------|
|    | CCC<br>Pro   | CAG<br>Gln | TTC<br>Phe        | GGC<br>Gly<br>140 | GGT<br>Gly                                  | CTG<br>Leu        | CCC<br>Pro        | GGC<br>Gly        | CAG<br>Gln<br>145 | CGC<br>Arg        | TAC<br>Tyr        | GGC<br>Gly        | GGC<br>Gly        | ATC<br>Ile<br>150 | TCG<br>Ser        | TCC<br>Ser        | 528 |      |
| 5  | CGC<br>Arg   | AAC<br>Asn | GAG<br>Glu<br>155 | TGC<br>Cys        | GAT<br>Asp                                  | CGG<br>Arg        | TTC<br>Phe        | CCC<br>Pro<br>160 | GAC<br>Asp        | GCC<br>Ala        | CTC<br>Leu        | AAG<br>Lys        | CCC<br>Pro<br>165 | GGC<br>Gly        | TGC<br>Cys        | TAC<br>Tyr        | 576 |      |
| 10 | TGG<br>Trp<br>170  | CGC<br>Arg | TTC<br>Phe        | GAC<br>Asp        | TGG<br>Trp                                  | TTC<br>Phe        | AAG<br>Lys<br>175 | AAC<br>Asn        | GCC<br>Ala        | GAC<br>Asp        | AAT<br>Asn        | CCG<br>Pro<br>180 | AGC<br>Ser        | TTC<br>Phe        | AGC<br>Ser        | TTC<br>Phe        | 624 |      |
| 15 | CGT<br>Arg<br>185  | CAG<br>Gln | GTC<br>Val        | CAG<br>Gln        | TGC<br>Cys                                  | CCA<br>Pro<br>190 | GCC<br>Ala        | GAG<br>Glu        | CTC<br>Leu        | GTC<br>Val        | GCT<br>Ala<br>195 | CGC<br>Arg        | ACC<br>Thr        | GGA<br>Gly        | TGC<br>Cys        | CGC<br>Arg<br>200 | 672 |      |
| 20 | CGC<br>Arg   | AAC<br>Asn | GAC<br>Asp        | GAC<br>Asp        | GGC<br>Gly<br>205                           | AAC<br>Asn        | TTC<br>Phe        | CCT<br>Pro        | GCC<br>Ala        | GTC<br>Val<br>210 | CAG<br>Gln        | ATC<br>Ile        | CCC<br>Pro        | TCC<br>Ser        | AGC<br>Ser<br>215 | AGC<br>Ser        | 720 |      |
|    | ACC<br>Thr   | AGC<br>Ser | TCT<br>Ser        | CCG<br>Pro<br>220 | GTC<br>Val                                  | AAC<br>Asn        | CAG<br>Gln        | CCT<br>Pro        | ACC<br>Thr<br>225 | AGC<br>Ser        | ACC<br>Thr        | AGC<br>Ser        | ACC<br>Thr        | ACG<br>Thr<br>230 | TCC<br>Ser        | ACC<br>Thr        | 768 |      |
| 25 | TCC<br>Ser   | ACC<br>Thr | ACC<br>Thr<br>235 | TCG<br>Ser        | AGC<br>Ser                                  | CCG<br>Pro        | CCA<br>Pro        | GTC<br>Val<br>240 | CAG<br>Gln        | CCT<br>Pro        | ACG<br>Thr        | ACT<br>Thr        | CCC<br>Pro<br>245 | AGC<br>Ser        | GGC<br>Gly        | TGC<br>Cys        | 816 |      |
| 30 | ACT<br>Thr<br>250  | GCT<br>Ala | GAG<br>Glu        | AGG<br>Arg        | TGG<br>Trp                                  | GCT<br>Ala        | CAG<br>Gln<br>255 | TGC<br>Cys        | GGC<br>Gly        | GGC<br>Gly        | AAT<br>Asn        | GGC<br>Gly<br>260 | TGG<br>Trp        | AGC<br>Ser        | GGC<br>Gly        | TGC<br>Cys        | 864 |      |
| 35 | ACC<br>Thr<br>265  | ACC<br>Thr | TGC<br>Cys        | GTC<br>Val        | GCT<br>Ala                                  | GGC<br>Gly<br>270 | AGC<br>Ser        | ACT<br>Thr        | TGC<br>Cys        | ACG<br>Thr        | AAG<br>Lys<br>275 | ATT<br>Ile        | AAT<br>Asn        | GAC<br>Asp        | TGG<br>Trp        | TAC<br>Tyr<br>280 | 912 |      |
| 40 | CAT<br>His   | CAG<br>Gln | TGC<br>Cys        | CTG<br>Leu        | TAGACGCAGG GCAGCTTGAG GGCCTTACTG GTGGCCGCAA |                   |                   |                   |                   |                   |                   |                   |                   |                   | 964               |                   |     |      |
|    | 285  |            |                   |                   |   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |     |      |
|    | CGAAATGACA CTCCCAATCA CTGTATTAGT TCTTGACAT AATTTGTC TCCCTCCAGG |            |                   |                   |   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |     | 1024 |
|    | GATTGTCACA TAAATGCAAT GAGGAACAAT GAGTAC                        |            |                   |                   |   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |     | 1060 |

45

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 305 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## 10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Arg Ser Ser Pro Leu Leu Pro Ser Ala Val Val Ala Ala Leu Pro  
 -21 -20 -15 -10

15 Val Leu Ala Leu Ala Ala Asp Gly Arg Ser Thr Arg Tyr Trp Asp Cys  
 -5 1 5 10

Cys Lys Pro Ser Cys Gly Trp Ala Lys Lys Ala Pro Val Asn Gln Pro  
 15 20 25

20 Val Phe Ser Cys Asn Ala Asn Phe Gln Arg Ile Thr Asp Phe Asp Ala  
 30 35 40

Lys Ser Gly Cys Glu Pro Gly Gly Val Ala Tyr Ser Cys Ala Asp Gln  
 25 45 50 55

Thr Pro Trp Ala Val Asn Asp Asp Phe Ala Leu Gly Phe Ala Ala Thr  
 60 65 70 75

30 Ser Ile Ala Gly Ser Asn Glu Ala Gly Trp Cys Cys Ala Cys Tyr Glu  
 80 85 90

Leu Thr Phe Thr Ser Gly Pro Val Ala Gly Lys Lys Met Val Val Gln  
 95 100 105

35 Ser Thr Ser Thr Gly Gly Asp Leu Gly Ser Asn His Phe Asp Leu Asn  
 110 115 120

Ile Pro Gly Gly Gly Val Gly Ile Phe Asp Gly Cys Thr Pro Gln Phe  
 40 125 130 135

Gly Gly Leu Pro Gly Gln Arg Tyr Gly Gly Ile Ser Ser Arg Asn Glu  
 140 145 150 155

45 Cys Asp Arg Phe Pro Asp Ala Leu Lys Pro Gly Cys Tyr Trp Arg Phe  
 160 165 170

Asp Trp Phe Lys Asn Ala Asp Asn Pro Ser Phe Ser Phe Arg Gln Val  
 175 180 185

50 Gln Cys Pro Ala Glu Leu Val Ala Arg Thr Gly Cys Arg Arg Asn Asp  
 190 195 200

Asp Gly Asn Phe Pro Ala Val Gln Ile Pro Ser Ser Ser Thr Ser Ser  
 55 205 210 215

47

Pro Val Asn Gln Pro Thr Ser Thr Ser Thr Thr Ser Thr Ser Thr Thr  
220 225 230 235

5 Ser Ser Pro Pro Val Gln Pro Thr Thr Pro Ser Gly Cys Thr Ala Glu  
240 245 250

Arg Trp Ala Gln Cys Gly Gly Asn Gly Trp Ser Gly Cys Thr Thr Cys  
255 260 265

10 Val Ala Gly Ser Thr Cys Thr Lys Ile Asn Asp Trp Tyr His Gln Cys  
270 275 280

15 Leu



## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 1473 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

10

## (iii) HYPOTHETICAL: NO

## (iv) ANTI-SENSE: NO

15

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: Fusarium oxysporum  
 (B) STRAIN: DSM 2672

## (ix) FEATURE:

20

- (A) NAME/KEY: CDS  
 (B) LOCATION: 97..1224

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

25

GAATTCGCGG CCGCTCATTC ACTTCATTCA TTCTTTAGAA TTACATACAC TCTCTTTCAA 60

AACAGTCACT CTTTAAACAA AACAACTTTT GCAACA ATG CGA TCT TAC ACT CTT 114  
 Met Arg Ser Tyr Thr Leu  
 1 5

30

CTC GCC CTG GCC GGC CCT CTC GCC GTG AGT GCT GCT TCT GGA AGC GGT 162  
 Leu Ala Leu Ala Gly Pro Leu Ala Val Ser Ala Ala Ser Gly Ser Gly  
 10 15 20

35

CAC TCT ACT CGA TAC TGG GAT TGC TGC AAG CCT TCT TGC TCT TGG AGC 210  
 His Ser Thr Arg Tyr Trp Asp Cys Cys Lys Pro Ser Cys Ser Trp Ser  
 25 30 35

40

GGA AAG GCT GCT GTC AAC GCC CCT GCT TTA ACT TGT GAT AAG AAC GAC 258  
 Gly Lys Ala Ala Val Asn Ala Pro Ala Leu Thr Cys Asp Lys Asn Asp  
 40 45 50

45

AAC CCC ATT TCC AAC ACC AAT GCT GTC AAC GGT TGT GAG GGT GGT GGT 306  
 Asn Pro Ile Ser Asn Thr Asn Ala Val Asn Gly Cys Glu Gly Gly Gly  
 55 60 65 70

50

TCT GCT TAT GCT TGC ACC AAC TAC TCT CCC TGG GCT GTC AAC GAT GAG 354  
 Ser Ala Tyr Ala Cys Thr Asn Tyr Ser Pro Trp Ala Val Asn Asp Glu  
 75 80 85

CTT GCC TAC GGT TTC GCT GCT ACC AAG ATC TCC GGT GGC TCC GAG GCC 402  
 Leu Ala Tyr Gly Phe Ala Ala Thr Lys Ile Ser Gly Gly Ser Glu Ala  
 90 95 100

55

|    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |      |
|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|------|
|    | AGC | TGG | TGC | TGT | GCT | TGC | TAT | GCT | TTG | ACC | TTC | ACC | ACT | GGC | CCC | GTC | 450  |
|    | Ser | Trp | Cys | Cys | Ala | Cys | Tyr | Ala | Leu | Thr | Phe | Thr | Thr | Gly | Pro | Val |      |
|    |     |     | 105 |     |     |     |     | 110 |     |     |     |     | 115 |     |     |     |      |
| 5  | AAG | GGC | AAG | AAG | ATG | ATC | GTC | CAG | TCC | ACC | AAC | ACT | GGA | GGT | GAT | CTC | 498  |
|    | Lys | Gly | Lys | Lys | Met | Ile | Val | Gln | Ser | Thr | Asn | Thr | Gly | Gly | Asp | Leu |      |
|    |     | 120 |     |     |     |     | 125 |     |     |     |     | 130 |     |     |     |     |      |
| 10 | GGC | GAC | AAC | CAC | TTC | GAT | CTC | ATG | ATG | CCC | GGC | GGT | GGT | GTC | GGT | ATC | 546  |
|    | Gly | Asp | Asn | His | Phe | Asp | Leu | Met | Met | Pro | Gly | Gly | Gly | Val | Gly | Ile |      |
|    | 135 |     |     |     |     | 140 |     |     |     |     | 145 |     |     |     |     | 150 |      |
| 15 | TTC | GAC | GGC | TGC | ACC | TCT | GAG | TTC | GGC | AAG | GCT | CTC | GGC | GGT | GCC | CAG | 594  |
|    | Phe | Asp | Gly | Cys | Thr | Ser | Glu | Phe | Gly | Lys | Ala | Leu | Gly | Gly | Ala | Gln |      |
|    |     |     |     |     | 155 |     |     |     |     | 160 |     |     |     |     | 165 |     |      |
| 20 | TAC | GGC | GGT | ATC | TCC | TCC | CGA | AGC | GAA | TGT | GAT | AGC | TAC | CCC | GAG | CTT | 642  |
|    | Tyr | Gly | Gly | Ile | Ser | Ser | Arg | Ser | Glu | Cys | Asp | Ser | Tyr | Pro | Glu | Leu |      |
|    |     |     |     | 170 |     |     |     |     | 175 |     |     |     |     | 180 |     |     |      |
| 25 | CTC | AAG | GAC | GGT | TGC | CAC | TGG | CGA | TTC | GAC | TGG | TTC | GAG | AAC | GCC | GAC | 690  |
|    | Leu | Lys | Asp | Gly | Cys | His | Trp | Arg | Phe | Asp | Trp | Phe | Glu | Asn | Ala | Asp |      |
|    |     |     | 185 |     |     |     | 190 |     |     |     |     |     | 195 |     |     |     |      |
| 30 | AAC | CCT | GAC | TTC | ACC | TTT | GAG | CAG | GTT | CAG | TGC | CCC | AAG | GCT | CTC | CTC | 738  |
|    | Asn | Pro | Asp | Phe | Thr | Phe | Glu | Gln | Val | Gln | Cys | Pro | Lys | Ala | Leu | Leu |      |
|    |     | 200 |     |     |     |     | 205 |     |     |     |     | 210 |     |     |     |     |      |
| 35 | GAC | ATC | AGT | GGA | TGC | AAG | CGT | GAT | GAC | GAC | TCC | AGC | TTC | CCT | GCC | TTC | 786  |
|    | Asp | Ile | Ser | Gly | Cys | Lys | Arg | Asp | Asp | Asp | Ser | Ser | Phe | Pro | Ala | Phe |      |
|    | 215 |     |     |     |     | 220 |     |     |     |     | 225 |     |     |     |     | 230 |      |
| 40 | AAG | GTT | GAT | ACC | TCG | GCC | AGC | AAG | CCC | CAG | CCC | TCC | AGC | TCC | GCT | AAG | 834  |
|    | Lys | Val | Asp | Thr | Ser | Ala | Ser | Lys | Pro | Gln | Pro | Ser | Ser | Ser | Ala | Lys |      |
|    |     |     |     |     | 235 |     |     |     |     | 240 |     |     |     |     | 245 |     |      |
| 45 | AAG | ACC | ACC | TCC | GCT | GCT | GCT | GCC | GCT | CAG | CCC | CAG | AAG | ACC | AAG | GAT | 882  |
|    | Lys | Thr | Thr | Ser | Ala | Ala | Ala | Ala | Ala | Gln | Pro | Gln | Lys | Thr | Lys | Asp |      |
|    |     |     |     | 250 |     |     |     | 255 |     |     |     |     |     | 260 |     |     |      |
| 50 | TCC | GCT | CCT | GTT | GTC | CAG | AAG | TCC | TCC | ACC | AAG | CCT | GCC | GCT | CAG | CCC | 930  |
|    | Ser | Ala | Pro | Val | Val | Gln | Lys | Ser | Ser | Thr | Lys | Pro | Ala | Ala | Gln | Pro |      |
|    |     |     | 265 |     |     |     | 270 |     |     |     |     |     | 275 |     |     |     |      |
| 55 | GAG | CCT | ACT | AAG | CCC | GCC | GAC | AAG | CCC | CAG | ACC | GAC | AAG | CCT | GTC | GCC | 978  |
|    | Glu | Pro | Thr | Lys | Pro | Ala | Asp | Lys | Pro | Gln | Thr | Asp | Lys | Pro | Val | Ala |      |
|    |     | 280 |     |     |     |     | 285 |     |     |     |     | 290 |     |     |     |     |      |
| 60 | ACC | AAG | CCT | GCT | GCT | ACC | AAG | CCC | GTC | CAA | CCT | GTC | AAC | AAG | CCC | AAG | 1026 |
|    | Thr | Lys | Pro | Ala | Ala | Thr | Lys | Pro | Val | Gln | Pro | Val | Asn | Lys | Pro | Lys |      |
|    |     | 295 |     |     |     | 300 |     |     |     |     | 305 |     |     |     |     | 310 |      |
| 65 | ACA | ACC | CAG | AAG | GTC | CGT | GGA | ACC | AAA | ACC | CGA | GGA | AGC | TGC | CCG | GCC | 1074 |
|    | Thr | Thr | Gln | Lys | Val | Arg | Gly | Thr | Lys | Thr | Arg | Gly | Ser | Cys | Pro | Ala |      |
|    |     |     |     |     | 315 |     |     |     |     | 320 |     |     |     |     | 325 |     |      |

50

|    |  |      |
|----|--|------|
|    | AAG ACT GAC GCT ACC GCC AAG GCC TCC GTT GTC CCT GCT TAT TAC CAG    | 1122 |
|    | Lys Thr Asp Ala Thr Ala Lys Ala Ser Val Val Pro Ala Tyr Tyr Gln    |      |
|    | 330 335 340  |      |
| 5  | TGT GGT GGT TCC AAG TCC GCT TAT CCC AAC GGC AAC CTC GCT TGC GCT    | 1170 |
|    | Cys Gly Gly Ser Lys Ser Ala Tyr Pro Asn Gly Asn Leu Ala Cys Ala    |      |
|    | 345 350 355  |      |
|    | ACT GGA AGC AAG TGT GTC AAG CAG AAC GAG TAC TAC TCC CAG TGT GTC    | 1218 |
| 10 | Thr Gly Ser Lys Cys Val Lys Gln Asn Glu Tyr Tyr Ser Gln Cys Val    |      |
|    | 360 365 370  |      |
|    | CCC AAC TAAATGGTAG ATCCATCGGT TGTGGAAGAG ACTATGCGTC TCAGAAGGGA     | 1274 |
|    | Pro Asn  |      |
| 15 | 375  |      |
|    | TCCTCTCATG AGCAGGCTTG TCATTGTATA GCATGGCATC CTGGACCAAG TGTTCGACCC  | 1334 |
|    | TTGTTGTACA TAGTATATCT TCATTGTATA TATTTAGACA CATAGATAGC CTCTTGTCAG  | 1394 |
| 20 | CGACAACCTGG CTACAAAAGA CTTGGCAGGC TTGTTCAATA TTGACACAGT TTCCTCCATA | 1454 |
|    | AAAAAAAAA AAAAAAAAAA   | 1473 |

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 376 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

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## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Arg Ser Tyr Thr Leu Leu Ala Leu Ala Gly Pro Leu Ala Val Ser  
 1 5 10 15  
 15 Ala Ala Ser Gly Ser Gly His Ser Thr Arg Tyr Trp Asp Cys Cys Lys  
 20 25 30  
 Pro Ser Cys Ser Trp Ser Gly Lys Ala Ala Val Asn Ala Pro Ala Leu  
 20 35 40 45  
 Thr Cys Asp Lys Asn Asp Asn Pro Ile Ser Asn Thr Asn Ala Val Asn  
 50 55 60  
 25 Gly Cys Glu Gly Gly Gly Ser Ala Tyr Ala Cys Thr Asn Tyr Ser Pro  
 65 70 75 80  
 Trp Ala Val Asn Asp Glu Leu Ala Tyr Gly Phe Ala Ala Thr Lys Ile  
 85 90 95  
 30 Ser Gly Gly Ser Glu Ala Ser Trp Cys Cys Ala Cys Tyr Ala Leu Thr  
 100 105 110  
 Phe Thr Thr Gly Pro Val Lys Gly Lys Lys Met Ile Val Gln Ser Thr  
 115 120 125  
 35 Asn Thr Gly Gly Asp Leu Gly Asp Asn His Phe Asp Leu Met Met Pro  
 130 135 140  
 40 Gly Gly Gly Val Gly Ile Phe Asp Gly Cys Thr Ser Glu Phe Gly Lys  
 145 150 155 160  
 Ala Leu Gly Gly Ala Gln Tyr Gly Gly Ile Ser Ser Arg Ser Glu Cys  
 165 170 175  
 45 Asp Ser Tyr Pro Glu Leu Leu Lys Asp Gly Cys His Trp Arg Phe Asp  
 180 185 190  
 Trp Phe Glu Asn Ala Asp Asn Pro Asp Phe Thr Phe Glu Gln Val Gln  
 195 200 205  
 Cys Pro Lys Ala Leu Leu Asp Ile Ser Gly Cys Lys Arg Asp Asp Asp  
 210 215 220  
 55 Ser Ser Phe Pro Ala Phe Lys Val Asp Thr Ser Ala Ser Lys Pro Gln  
 225 230 235 240



FIG. 1

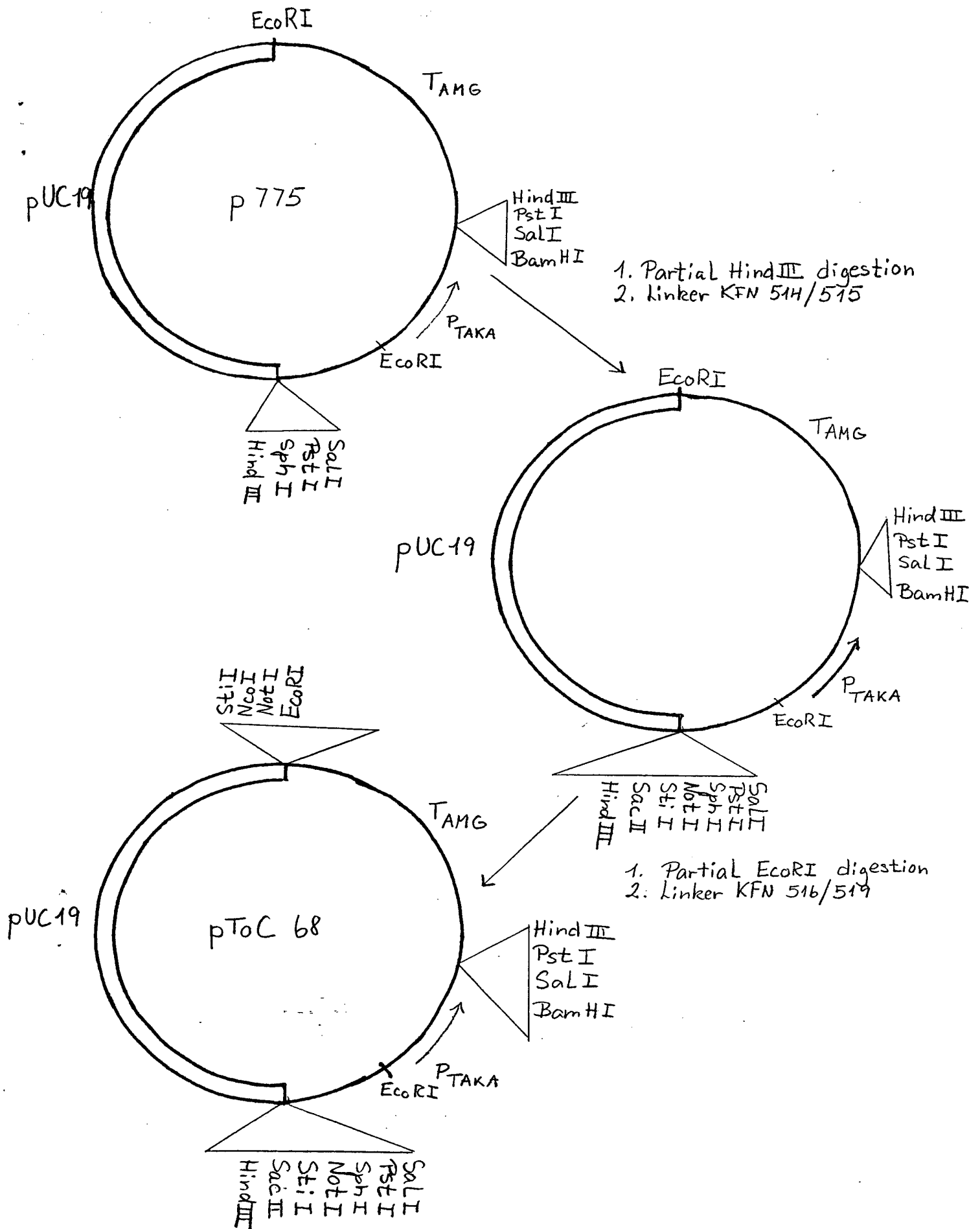


FIG. 2

